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Effects of a whey protein supplementation on oxidative stress, body composition and glucose metabolism among overweight people affected by diabetes mellitus or impaired fasting glucose: A pilot study

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Abstract

Obesity and diabetes mellitus type 2 (DM2) are characterized by chronic inflammation and oxidative stress [Donath et al. 2013] and this leads to cardiovascular diseases [Hulsmans & Holvoet 2010]. Whey proteins (WP) have antioxidant [Chitapanarux et al. 2009], anti-inflammatory [Sugawara et al. 2012] and hypoglycemic activities [Mignone et al. 2015], while data on weight, body composition [Frestedt et al. 2008; Aldrich et al. 2011] and blood pressure are conflicting [Kawase et al. 2000; Lee et al. 2007]. WP have unpleasant taste and smell [Patel 2015], but a new WP isolate (ProLYOtin®) seems to be more palatable. 40 g/die of ProLYOtin® were supplemented to overweight people (n=31) with impaired fasting glucose/DM2 for 12 weeks. Markers of antioxidant status (total antioxidant status, glutathione peroxidase, glutathione reductase, uric acid), oxidative damage (thiobarbituric acid reactive substances, advanced oxidation protein products, 8-hydroxydeoxyguanosine), inflammation (interleukin-6, high sensitive reactive protein C) and glicemic status (fasting glucose, insulin, glycated hemoglobin), anthropometric data (weight, height, waist circumference), body composition (body cell mass, fat mass), blood pressure, hand grip strength and skin autofluorescence were measured before and at the end of supplementation. Isolate palatability was evaluated. An increase in glutathione peroxidase, a decrease in uric acid and no change in glutathione reductase, total antioxidant status, oxidative damage, inflammation and glucose markers were found. Significant improvements in anthropometric parameters and fat mass were detected. There wasn't any change in blood pressure, skin autofluorescence and physical performance. Two-thirds of subjects judged the supplement positively. ProLYOtin® seems suitable for treatment of OS and overweight.

Keywords: Whey proteins; Oxidative stress; Body composition; Glucose metabolism; Skin autofluorescence; Palatability

1. Introduction

The incidence and the prevalence of overweight and obesity are increasing and diabetes mellitus type 2 (DM2) is rising accordingly [1]. People with visceral obesity have a high risk to develop metabolic syndrome (MS), *i.e.* a condition characterized by increased waist circumference, hypertriglyceridemia, low HDL, hypertension and impaired fasting glucose (IFG)/DM2 [2]. Metabolic syndrome and its components have a manifold pathogenesis; however it has been assessed that insulin resistance is a main cause of them [3].

Visceral obesity and DM2 are characterized by chronic inflammation and high levels of oxidative stress (OS) [4]. OS is a disruption in the balance between oxidative and reductive reactions [5], plays a pivotal role in the development of cardiovascular diseases and is caused by visceral obesity and DM2 [6–9].

Besides different drugs, several functional foods have been investigated in order to find new treatment strategies for MS determinants and whey proteins (WP) appeared very promising [10].

Whey is the liquid remaining after precipitation and removal of milk casein curd during cheese manufacture [11]. WP represent the protein fraction of whey [11] and an heterogeneous group of molecules: β-lactoglobulin (50%, β-Lg), glycomacropeptide (20–25%, GMP), α-lactoalbumin (20%, α-La), bovine serum albumin (5%, BSA), immunoglobulins (1–2%, Ig), lactoferrin (LF), lactoperoxidase (LP) and WP minor proteins [12,13].

WP is rich in essential and branched-chain amino acids (BCAAs) [11] and rich in cysteine which represents the building block of glutathione (GSH) [14], the main intracellular antioxidant [15].

GSH regulates intracellular levels of reactive oxygen species (ROS) and has immunostimulant activity [15,16]. Both animal and clinical studies showed that WP can increase GSH levels [17–24]. WP was able to increase total antioxidant capacity (TAS) and antioxidant enzymes [19,25] and reduce OS markers as TBARS [20,22,25].

WP seems to induce glutathione peroxidase (GPX) gene expression [26,27].

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Animal studies showed that WP can reduce levels of proinflammatory cytokines like IL-6, TNF-α [20,28,29,30] and increase those of anti-inflammatory ones, IL-2, IL-4, IL-7 and IL-8 [30]. However, human studies revealed conflicting results on WP capacity to reduce inflammatory cytokines [21,31–33] and C-reactive protein (CRP) [31–34]. WP could eradicate HCV, reduce ALT and increase IL-2 levels and natural killer cells (NK) activity in adults affected by viral hepatitis [35–37] and exert anti-inflammatory activities modulating adipocytes autocrine/paracrine renin-angiotensin system [38–40].

When added to meals, WP reduce post-meal glycemia as reviewed in Mignone et al. 2015 [41] and Adams & Broughton 2016 [42]. WP amino acids, and in particular BCAAs, stimulate directly insulin secretion [43–45], while WP peptides elicit gastric inhibitory peptide (GIP) release [46–48]. GIP is an enteric hormone, called, together with the glucose-dependent insulinotropic peptide (GIP), incretin, that stimulates pancreatic β-cells [41,42]. WP are more powerful than glucose and fructose in stimulating intestinal incretin release [49]. WP exerts these effects in health and DM2 and they are more powerful when administered before meals [44,50,51]. However, conflicting data have been highlighted about WP capacity to induce GLP-1 secretion in DM2 patients [43,44,52]. WP also stimulate a gastro-pyloric feedback which inhibits gastric empting [53,54] and increase GLP-1, cholecystokinin (CCK) and peptide YY (PYY) levels, that delay stomach empting themselves [41]. WP are in general more powerful than other proteins in stimulating gastroenteric hormones [31,46,47,50,55–57]. WP proved also to be able to inhibit α-glucosidase, that mediates intestinal glucose absorption [41], and dipeptidil peptidase-IV (DPP-IV), an enzyme that degrades incretins [41,42]. WP proved to be effective in improving glycemic control among healthy and diabetic patients and could be a new future therapeutic approach for DM2 [41,42].

Clinical studies show that WP administered before meals increase sense of satiety and reduce food intake [41,58,59] and that they are more powerful than other types of proteins in this last regard [46,60,61]. Direct and indirect mechanisms on central nervous system and have been advocated. WP amino acids post-meal plasma peak could suppress appetite through a vagal feedback and a direct effect on hypothalamus [46,41,62–65]. Moreover, trytophan plasma peak seems to increase serotonin (5-HT) production [41]. Gastroenteric hormones above mentioned have also anorexigenic effects. WP suppress ghrelin which has an orexigenic action [41,66]. In addition, WP could influence sense of satiety by modulation of hepatic lipogenesis [67].

WP effect on body composition was tested among overweight people alone and in association to weight loss programs. Some studies have shown that chronic WP supplementation can reduce fat mass (FM), reduce/maintain body weight and preserve/improve lean muscular [68–70]. On the contrary, two other researches didn't find any effect of WP on weight and body composition [31,71].

WP reduce FM and increase muscular protein synthesis more than soy proteins during low-calorie diets [72,73]. Tahavorgar and colleagues demonstrated that a whey poteen concentrate (65 g) was more effective than a soy protein supplement (60 g) in improving body mass index (BMI), waist circumference, FM and lean muscular mass [61]. Moreover, WP were more powerful than casein and soy proteins in increasing energy expenditure through improved thermogenesis [41] and reducing body weight and FM [74].

Hypertension is a main cardiovascular risk factor [75] and several studies suggested that WP have antihypertensive properties and improve vascular function. However, up to now, studies that investigated acute postprandial effect of WP on blood pressure and vascular function highlighted conflicting results [32,76,77]. Also, results from studies investigating the antihypertensive activity of WP chronic supplementation were inconsistent [69,78–81].

WP are formulated in three main forms: concentrate (WPC, 35–80% proteins), isolate (WPI, 85–90% proteins) and hydrolysate (WPH, 100% proteins) [41]. They are commercialized as nutritional supplement since decades [13] and no adverse effects after their administration have been reported in the literature [82,83].

However, big limits for WP use in clinical practice are their unpleasant taste [84–86] and odor [87]. These drawbacks are caused by low molecular weight metabolites of enzymatic hydrolysis [88] and lipid oxidation products [89] and could lead to low therapeutic compliance. WPC have usually the worst palatability [41].

The aim of this study was testing the effects of ProLYOtin®, a new WPI with a pleasant taste, on markers of antioxidant status and oxidative damage, glucose metabolism, BMI, body composition, muscular performance, markers of glycosilation tissue damage and blood pressure among overweight/obese people affected by DM2 or IFG. Finally, the palatability of the supplement was evaluated.

2. Materials and methods

2.1. Study subjects

Overweight/obese patients affected by DM2 or IFG attending the Dietetics and Clinical Nutrition Unit of Bolzano District Hospital (Italy) during 2014–2015 were offered to participate to the present study. 34 adult subjects were enrolled and divided into 2 groups according to their HbA1c values: group A HbA1c between 6.0% and 6.4% (n=15), group B HbA1c between 6.5% and 10% (n=19). Main exclusion criteria were: ongoing therapy with insulin, glucocorticoid or immunosuppressant drugs, end stage renal disease (ESRD, eGFR <60 ml/min/1.73m^2), nephrotic syndrome, allergy to milk proteins, intestinal malabsorption, cystic fibrosis, stage Child B and C liver cirrhosis, severe psychopathology, glucose-6-phosphate-dehydrogenase deficiency, pregnancy, breast feeding and inability to consent to participate. Subjects belonging to group A should not have taken any hypoglycemic drug in the previous 3 months to and during the study period, while subjects belonging to group B should not have changed hypoglycemic therapy in the previous 3 months to and during the study period. Finally, diabetes duration in group B should have lasted more than 12 months and less than 15 years. Being the present study a pilot, a small sample size was accepted to test the effect and the palatability of this new WPI.

Table 1 shows characteristics of subjects who completed the experiment at baseline.

All patients were following a moderate personalized hypocaloric, normoprotein diet.

The study was approved by local ethical committee (Ethics committee meeting on 17th April 2013, Approval n. 24) and informed consent was obtained from all participants.

2.2. WP supplement characteristics

The supplement (ProLYOtin®) is a WPI with 90% of proteins and was produced with chromatographic and ceramic membrane filtration techniques. ProLYOtin® Biological Value is between 120 and 130, while it's Digestible Indispensable Amino Acid Score is 1.25. In Tables 2 and 3, the amino acidic profile and average composition are displayed.

2.3. Experimental design

Patients were administered 20 g of ProLYOtin® powder mixed with water 30′ before lunch and 30′ before dinner (40 g/die) for 12 weeks. Data and samples were collected at Time 0 (T0=before supplementation) and at Time 12 (T12=after 12 weeks of supplementation) in fasting condition. At the end of the study subjects also evaluated supplement palatability on a specific scale. In case of drop-out volunteers were asked to fill a form in order to assess the reason for that.

Table 1
Characteristics of patients completing the study at baseline (ns=non statistically different, P<.05; s=statistically different P>.05)

	All (n=31)	Group A (n=13)	Group B (n=18)	Difference among groups
Age (years)	55.7±8.5	57.2±7.3	54.6±9.3	ns
Height (cm)	164±9	162±10	165±8	ns
Weight (kg)	100.1±24.4	93.3±19.4	105.0±26.9	ns
BMI (kg/m^2)	37.1±7.9	35.5±6.2	38.3±9.0	ns
Waist circumference (cm)	115.4±17.7	110.8±15.1	118.7±19.1	ns
% female	61.3%	69.2%	55.6%	ns
% smokers	12.9%	0.0%	22.2%	ns
Blood pressure systolic (mmHg)	133.3±17.2	132.5±20.4	133.9±15	ns
Blood pressure diastolic (mmHg)	83.4±11.9	81.4±11.5	84.8±12.3	ns

Table 2
Amino acidic profile of ProLYOtin® (** BCAAs)

Amino acid	g/100 g
Alanin	4.95
Arginin	1.86
Aspartic acid	10.59
Cystein	2.53
Glutamic acid	16.67
Glycine	1.51
Histidin	1.47
Isoleucine**	6.01
Leucin**	10.21
Lysin	8.86
Methionine	2.06
Phenylalanin	2.79
Proline	5.05
Serine	4.25
Threonine	6.55
Tryptophan	1.81
Tyrosin	2.77
Valin**	5.48

Table 3
Average composition of ProLYOtin®

Protein	%
β-lactoglobulin	46.2–52.8
α-lactoalbumin	24.1–27.8
Immunoglobulins	5.8–8.8
Bovine serum albumin	4.9–8.6
Glycomacropeptide	1.8–2.2
Lactoferrin	0.6–0.9
Lactoperoxidase	0.4–0.7
Casein	0
Fats	0
Lipoproteins	0

2.4. Blood and urine sampling

Venous blood samples were collected from each patient using vacuum sealed tubes (Vacutainer©) and immediately centrifuged at 3000 rpm for 10 min at RT. First morning spot urine samples were also collected. Plasma, urine and serum samples were stored at −80 °C until analysis according to analyte stability.

2.5. Biochemical tests

EDTA anticoagulant tubes were used for the measurement of glycosilated hemoglobin (HbA1C); heparinized whole blood was used for the measurement of Glutathione Peroxidase (GPx); plasma samples were used for measurement of biochemical parameters (uric acid, IL 6, PCR, glucose and insulin), for Glutathione Reductase (GR) and for Total Antioxidant Status (TAS); serum samples were used for Advanced Oxidation Protein Products (AOPP) assay; urine samples were used for measurement of Thiobarbituric Acid Reactive Substances (TBARS) and for 8-Hydroxydeoxyguanosine (8-OhdG).

Glutathione Peroxidase (GPx) activity was measured in heparinized whole blood using commercial test kit (Randox®, UK, 2013) based on the procedure of Paglia and Valentine [90]. GPx catalyzes the oxidation of Glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured. Glutathione Reductase (GR) activity was measured in plasma using commercial test kit (Randox®, UK, 2010). GR catalyzes the reduction of Glutathione (GSSG) in the presence of NADPH, which is oxidized to $NADP^+$. The decrease in absorbance at 340 nm was measured. Total Antioxidant Status (TAS) was measured in plasma using commercial test kit (Randox®, UK, 2013). ABTS® (2,2-Azino-di-[3-ethylbenzthiazoline sulphonate]) was incubated with a peroxidase (metmyglobin) and H_2O_2 to produce the radical cation ABTS®$^{*+}$. This has a relatively stable blue-green color, which was measured at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which is proportional to their concentration. The advanced oxidation protein products (AOPP) assay was measured in serum samples using OxiSelect™ AOPP Assay kit (Cell Biolabs, Inc.©, USA, 2007–2014). The unknown AOPP-containing samples were first mixed with an assay reaction initiator that began a color development process. After a brief incubation, a stop solution were added and the samples and standards were read at 340 nm with a standard colorimetric plate reader. Thiobarbituric Acid Reactive Substances (TBARS) assay were measured in urine samples using the colorimetric assay by Cayman Chemical Company©, USA (2013). Malondialdehyde is a naturally occurring product of lipid peroxidation. The MDA-TBA adduct formed by the reaction of MDA and TBA under high temperature (90–100 °C) and acidic conditions was measured colorimetrically at 530–540 nm. 8-Hydroxydeoxyguanosine (8-OhdG) assay was measured in urine samples using HPLC analytical separations with electrochemical detection (HPLC-ED) as described by Bolner and colleagues [91].

Homeostasis model assessment-insulin resistance (HOMA-IR), a method for assessing insulin resistance from fasting plasma glucose and insulin levels [92], was calculated using the equation: [Fasting plasma glucose (mg/dl) × fasting plasma insulin (mcUI/ml)]/405 [93].

2.6. Anthropometric parameters, hand grip strength and blood pressure

Body weight and height were determined while subjects were wearing light clothing and no shoes with a digital medical scale integrated with a mechanical measuring rod (Seca 910, Seca, Switzerland). Body mass index (BMI=weight (kg)/height (m^2)) was accordingly calculated. Waist circumference (the midway in the distance of the superior iliac crest and the lower margin of the last rib in the horizontal plane [94]) was registered with a flexible meter.

Muscular performance was estimated as peak hand grip strength with an isometric dynamometer (Jamar® Hydraulic Hand Dynamometer, Sammons Preston Inc., IL, USA), in sitting and standing position with the elbow at a 90° angled position. Both hands were tested.

Blood pressure was measured with a mercury sphygmomanometer (Erkameter 3000, Erka, Bad Tölz, Germany) using a "large adult size" cuff and a stethoscope (3M^{MT} Littmann^{MT} Classic II SE) with Riva Rocci's method.

2.7. Skin autofluorescence

Skin autofluorescence (AF), that is a noninvasive measure for tissue advanced glycation end products (AGEs) and cumulative metabolic stress [6,95], was measured with AGE – Reader SU (DiagnOptics Technologies BV, Groningen, Netherlands) at T0 and T12. This methodology was already described previously in the literature [95].

AGE Reader Software 2.3.0 received and analyzed immediately the data on the computer. Mean skin AF was calculated from three consecutive measurements.

Table 4
Variations of biochemical parameters for pooled subjects

Category	Parameter	T0 (mean ± S.D.)	T12 (mean ± S.D.)	Difference (%)	p
Antioxidant status	Glutathione – reductase (GR) (U/l)	65.87±12.48	62.77±12.97	4.70	0.2975
	Glutathione – peroxidase (GPX) (U/l)	4819.16±1627.61	5699.13±1938.70	−18.26	**0.0053**
	Uric acid (mg/dl)	6.07±1.44	5.76±1.16	5.05	**0.0350**
	Total antioxidant status (TAS) (mmol/l)	1.54±0.14	1.55±.033	−0.88	0.8151
Oxidative damage	Thiobarbituric acid reactive substances (TBARS) (μmol/g)	0.67±0.81	0.56±0.45	16.87	0.3389
	Thiobarbituric acid reactive substances (TBARS) (μmol/g creat)	5.13±6.57	4.60±3.43	10.28	0.5211
	Advanced oxidation protein products (AOPP) (μM/Chlor-T-eq)	322.67±111.57	313,89±115,50	2.72	0.6924
	Urinary 8-hydroxydeoxyguanosine (8OHdG) (mg/g creat)	162.45±204.84	188.00±207.46	−15.73	0.4441
Inflammatory state	IL-6 (pg/ml)	3.83±2.29	3.50±2.29	8.65	0.3314
	PCR (mg/dl)	0.48±0.44	0.50±0.58	−3.00	0.8308
Metabolic parameters	Glucose (mg/dl)	118.89±35.25	114.32±22.76	3.84	0.3111
	Insulin (mcUI/ml)	18.10±8.54	17.59±10.16	2.82	0.6854
	HbA1c (%)	6.84±1.00	6.66±0.76	2.59	0.1369
	HOMA-IR	5.42±3.42	5.16±3.85	4.83	0.6421

Table 5
Variations of anthropometric parameters for pooled subjects

Anthropometric parameter	T0 (mean ± S.D.)	T12 (mean ± S.D.)	Difference (%)	p
Weight (kg)	100.1±24.4	96.7±21	3.44	**0.0066**
BMI (kg/m^2)	37.1±7.9	35.9±6.7	3.37	**0.0058**
Waist circumference (cm)	115.4±17.7	112.2±16.5	2.76	**0.0065**
Systolic blood pressure (mmHg)	133.3±17.2	131.9±13.9	1.06	0.6590
Diastolic blood pressure (mmHg)	83.4±11.9	83.9±9.5	−0.66	0.7834

Table 6
Variation of body composition for pooled subjects

Body compartments	T0 (mean ± S.D.)	T12 (mean ± S.D.)	Difference (%)	p
FM (kg)	42.59±16.32	38.83±13.69	8.84	**0.0003**
FM (%)	41.71±8.85	39.45±9.18	5.41	**<0.001**
BCM (kg)	29.18±7.98	28.64±7.95	1.87	0.1527
BCM (%)	50.13±5.14	49.29±4.82	1.67	**0.0389**

Table 7
Variation of hand grip strength in sitting and standing positions in the nondominant (ND) and dominant arm (D) for pooled subjects

Hand grip (kg)	T0 (mean ± S.D.)	T12 (mean ± S.D.)	Difference (%)	p
ND arm – sitting	32.19±11.45	32.47±10.35	−0.85	0.7915
ND arm – standing	32.10±11.41	32.90±10.53	−2.49	0.3200
D arm – sitting	33.42±12.25	33.85±12.14	−1.31	0.6761
D arm – standing	33.77±13.59	34.95±12.91	−3.50	0.3212

2.8. Bioelectrical impedance analysis

FM and body cell mass (BCM) were measured by bioelectrical impedance analysis (BIA), a non-invasive method to estimate body composition [96]. Resistance (R) and reactance (Xc) were measured with the bioimpedance analyzer BIA 101 (Akern, Florence, Italy) positioning two electrodes on the left hand (metacarpal-phalangeal and radio-ulnar joints) and two electrodes on the left foot (metatarsal-phalangeal and ankle joints) according to the ESPEN guidelines for Bioimpedance analysis [97]. Body compartments were then estimated by means of predictive equations with a software (BodyGram Pro 3.0). Predictive equations were also based on weight, height, sex and age.

2.9. Palatability scale and tolerance

In order to examine ProLYOtin® palatability, volunteers answered the following question "*How did you like the whey protein supplement?*" using a 7 point hedonic scale (1=extremely unpalatable, 2=moderately unpalatable, 3=slightly unpalatable, 4=neither unpalatable nor palatable, 5=slightly palatable, 6=moderately palatable, 7=extremely palatable).

Patients were instructed to report side effects.

2.10. Statistical analyses

An Excel datasheet (Microsoft Corporation, Santa Rosa, CA, USA) was compiled and used for statistical calculations like mean, S.D. and range. Data were analyzed using Prism 6 (Graphpad Software, La Jolla, CA, USA). Parameters detected at T0 were compared with those collected at T12 among all participants and in the 2 groups separately by a paired *t* test and a $p<0.05$ was considered significant.

3. Results

There was one drop-out in group A and one subjects in every group was excluded from analyses because they took less than 2/3 of the total amount of ProLYOtin® (31 completers; group A $n=13$, group B $n=18$). The subject who abandoned the study never filled the drop-out form. Moreover a completer didn't evaluate the palatability of ProLYOtin®.

3.1. Biochemical parameters

There wasn't any statistically significant difference in biochemical parameters between T0 and T12 for pooled subjects except for GPX (mean difference−18.26%, $p=0.0053$) and uric acid (mean difference 5.05%, $p=0.0350$) (Table 4).

When date was analyzed in group A and B separately, only a significant difference in acid uric in group A (mean difference 7.65%, $p=0.0314$) and in GPX in group B mean difference (mean difference −15.54%, $p=0.0368$) were found (Appendix, Table 1).

Examining data according to gender, a statistically significant difference between T0 and T12 was found for GPX (mean difference −22.19%, $p=0.0091$) among females, while no differences were detected among males (Appendix Table 1).

3.2. Anthropometric data

A statistically significant difference between T0 and T12 rose for weight (average mean difference 3.44%, $p=0.0066$), BMI (mean difference 3.37%, $p=0.0058$) and waist circumference (mean difference 2.76%, $p=0.0065$) (Table 5).

When anthropometric data were examined in group A no differences were found between T0 and T12 for all parameters (Appendix Table 1).

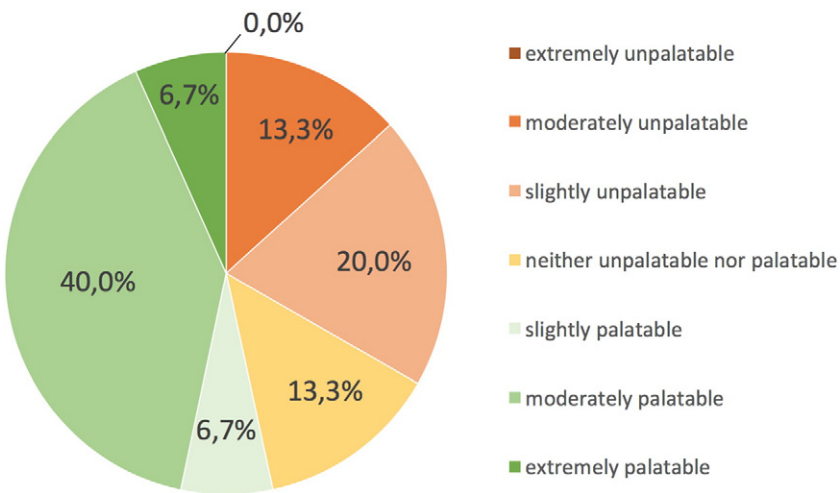

Fig. 1. ProLYOtin® palatability.

In group B there was a statistically significant change only in BMI (mean difference 3.42%, $p=0.0484$) and waist circumference (mean difference 2.60%, $p=0.0136$) (Appendix Table 1).

Among females there was a statistically significant variation in BMI (mean difference 3.14%, $p=0.0457$), while among males there was a change in waist circumference (mean difference 3.70%, $p=0.0380$) (Appendix Table 1).

3.3. Body composition

A significant change in absolute FM (average mean difference 8.84%, $p=0.0003$), percent FM (5.41, $p=0.0000$) and percent BCM (average mean difference 1.67%, $p=0.0389$) was detected in pooled subjects at the end of the study (Table 6).

In group A there was a statistically significant variation in absolute FM (mean difference 8.50%, $p=0.0039$), percent FM (mean difference 5.05%, $p=0.0050$). The same results were detected in group B: absolute FM (mean difference 9.05%, $p=0.0141$), percent FM (mean difference 5.68%, $p=0.0020$) (Appendix Table 1).

A significant difference was found for absolute FM (mean difference 6.95%, $p=0.0261$) and percent FM (mean difference 3.40%, $p=0.0040$) among females. Male absolute FM (mean difference 12.65%, $p=0.0026$), percent FM (mean difference 9.76%, $p=0.0017$) and percent BCM (mean difference 2.67%, $p=0.0138$) decreased at the end of the study (Appendix Table 1).

3.4. Muscular performance

No statistically significant difference was found in hand grip strength in both arms and measurement conditions for pooled (Table 7), group A and group B subjects (Appendix Table 1). The same results were detected also examining data according to gender (Appendix Table 1).

3.5. Skin autofluorescence

We didn't find any difference in AF in pooled subjects (T0 2.56±0.43, T12 2.63±0.49, mean difference−2.68%, $p=0.3401$). The same results were detected in group A, group B (Appendix Table 1). On the contrary when volunteers were divided by sex there was a statistically significant change in AF among female (T0 2.49±0.35, T12 2.69±0.47, mean difference−8.16%, $p=0.0098$) and male (T0 2.74±0.58, T12 2.49±0.53, mean difference 9.37%, $p=0.0383$) (Appendix Table 1).

3.6. Palatability and tolerance

Among patients who completed the study and filled the palatability scale, 0% judged the supplement extremely unpalatable, 13.3% moderately unpalatable, 20% slightly unpalatable, 13.3% neither unpalatable nor palatable, 6.67% slightly palatable, 40% moderately palatable, 6.67% extremely palatable; Fig. 1).

There were no side effects of the therapy during the supplementation.

4. Discussion

The present study showed that a 12 weeks supplementation with a new WPI (20 g preprandem, BID) in patients with IFG or T2DM and overweight could increase GPX levels, decrease uric acid, while it didn't exert any effect on GR and TAS. These results were substantially confirmed also in the subgroups analysis.

GPX is an enzyme that catalyzes the GSH-dependent reduction of many peroxides. GPX expression is regulated by ROS levels *via* the Nrf/Keap1 system. When ROS rises Keap is oxidized and cannot bind Nrf anymore. Nrf migrates in the nucleus and binds to ARE (EpRE) DNA element of target genes including that encoding for GPX and induces its transcription [98]. WP are rich in cysteine that is the building block for GSH and therefore they should improve OS [14,17–19,23]. Consequently GPX, expression should be reduced after WP supplementation and this is in contrast with our results. However, one study demonstrated that a WPH administered to mice with hepato- and nephropathy increased GPX in their liver homogenate [25] and two studies highlighted that WP, alone or in association with other types of antioxidants, could induce gene expression of GPX [26,27]. Moreover, GPX is a selenoprotein as it contains a selenocysteine residue in its active site [98]. Selenium (Se) concentrations in WP is around 217–457 ng Se/g [99] and this element is mainly bond to high molecular weight species [100]. The external supply of Se with ProLYOtin® could have increased GPX synthesis. Finally, among WP minor proteins there is also a little amount of GPX [101]. Accordingly, the GPX increase we found could be due to its enhanced synthesis stimulated by WP and its, although little, external introduction.

Tiol depletion usually reduces GR activity, the enzyme that reconverts GSSG in GSH, and to our knowledge just one study tested WP effect on GR in the past showing positive results [102]. Our supplementation unfortunately didn't show any outcome on this parameter.

Uric acid is an end-product of purine nucleotides metabolism [103,104] and is known as a powerful antioxidant as it acts as free radical scavenger and a chelator of transitional metal ions [103]. However, it also acts as a pro-oxidant as ROS are byproducts of its production. Moreover, uric acid could have pro-oxidants actions through the urate radical or by stimulating the synthesis of proinflammatory molecules [104]. Hyperuricemia is usually associated with OS and interpreted as a protective mechanism against its damage [104]. Elevated serum acid levels are independently associated with risk of hypertension, DM2 and cardiovascular disease [105]. The reduction of acid uric found in the present study could be sign of an improvement of the global metabolic-oxidative state due to WP.

TAS measures the global activity of antioxidant enzymes and differently to previous studies WP didn't exert any effect on it [19].

This new WPI didn't exert any significant effect on oxidative damage markers (TBARS, AOPP and 8-OhdG) both among pooled subjects and subgroups. Moreover, there wasn't any consistent change tendency in the parameters, as TBARS and AOPP appeared to decrease while 8-OhdG appeared to increase. These data are in contrast with the pre-existing literature [20,22,25].

Looking at the whole picture, WPI had a limited effect on oxidative damage markers and antioxidant enzymes, except for GPX and uric acid. This could be due to sample size issues. Other variables that should be taken into account are the dosage and the duration of the supplementation needed in order to elicit an effect on OS in humans as most of the studies were conducted on animal models [19,20,22,25]. In addition, variation in physical activity, that influences GSH [106] and antioxidant enzymes [107] levels, could have biased our results.

We didn't observe any statistically significant change in IL-6 and PCR levels. The absence of any variation agrees with some data present in the literature on anti-inflammatory effects of WP in humans [31,32], but disagrees with some other [21,33,34]. In this case we cannot impute the disparity in the results to the amount of WP administered or the duration of the supplementation as our protocol was in line or more “heavy” than those of other studies [21,31–34]. A possible explanation to these conflicting data could be the employment of different forms of WP (WPI, WPH, WPC).

Our results didn't show any significant effect of this new WPI on blood glucose, insulin-levels, HOMA-I and HbA1c. This is in conflict with the existing pieces of knowledge about WP activities on glucose metabolism. In the past WP amino acids proved to be able to increase insulin release directly and indirectly acting on GIP, DPP-IV and α-glucosidase. They can also slow gastric empting through direct and

indirect mechanisms and reduce satiety [41–46,50,51]. However, it is worth to notice that there was a tendency for improvement for all of them (especially HbA1c) and our results could be explained by a too small sample size to reach statistical significance. Dosage and duration of the supplementation was not inferior to those of previous studies. GLP-1 release could be reduced in patients with long lasting DM2 [43,44,52] and WPI could not have exerted a strong incretin effect at least in group B because of this reason.

At the end of the study any significant difference in AF was detected. To our knowledge no other study evaluated WP effect on this parameter, that is an indicator of glyco-oxidative processes. In particular it represents the levels of advanced glycation end products (AGEs) bond to proteins with slow turnover (e.g., collagen) in the skin [6]. This result is in line with our negative findings on OS and glycemic metabolism. However, it could also due the relative short duration of our study in comparison to skin-collagen half-life (15 years) [95,108]. Gender subgroup analysis showed a significant increase in AF in females and a significant decrease in males. More studies are probably needed to clarify the significance of this datum, as both females and males showed no change in glucose metabolism and little change in OS parameters.

The study showed significant improvements in body weight, BMI, waist circumference, FM (both percent and absolute) and %BCM among pooled subjects. Similar findings were found also in subgroup analysis. It seems that body composition and weight changes were due to a reduction in FM and possibly visceral fat. These results are in line with some previous studies [68,69,72,74], but contradict some other [31,72]. A possible explanation to these conflicting data could be the employment of different forms of WP and supplementation modalities.

Absolute BCM didn't change as hand grip strength didn't. Several studies in the past found positive effects on muscular mass [69,70] and performance [109] after WP supplementation. However, one detected a null effect of WP on muscular mass during a weight loss program [68]. During weight loss programs, that aim to reduce fat mass, also a part of muscle mass is lost [110]. As study subject were on hypocaloric diets in the present research we could interpret this datum positively as a “sparing effect” of WPI on muscle.

In the past, studies that investigated chronic effect of WP on blood pressure in humans highlighted conflicting results [69,78–81]. No differences in BP were detected in our work even if dosage and duration of the supplementation were similar to those of previous studies. More investigations are probably necessary in order to understand the real antihypertensive power of WP in clinical settings.

Another hypothesis to explain our puzzling results is that the new isolation process altered the biochemical and physiological properties of whey. However, this seems to be unlikely to us as during production proteins were not exposed to high temperatures.

The similarity of results between group A and B (Appendix Table 1) is not surprising considering that IFG and DM2 represent simply two different stages of the same metabolic disturbance. As DM2, IFG is a condition associated to the excess of adipose tissue in which the body is not able to control properly blood sugar levels. HbA1c is >6%, but it doesn't reach 6.5%. Usually, when not properly treated with a change of life-style, IFG evolves into DM2 [2]. These similar results can be also be explained by the fact that HbA1c difference between the two groups at the beginning of the study was not striking (Group A: HbA1c 6.22%, Group B: HbA1c 7.29%, $p=0.002$).

With regard to palatability, differently to previous findings [84–86], we found positive results. Indeed, most of the people rated ProLYOtin positively: 33.3% judged it unpalatable, while 66.7% rated it palatable or neither unpalatable nor palatable. As we tested a supplement with clinical applications (and not a dish!) the answer “nor palatable nor unpalatable” must be considered positively. As our new formula was well tolerated and well accepted, it could be in the future very suitable for clinical application.

To sum up, a new WPI supplemented for 12 weeks in overweight people affected by IFG/DM2 could improve some OS markers (GPX and uric acid) and body composition, but could not meliorate inflammatory indices, skin AF, glucose metabolism and blood pressure. This formula was also well accepted by volunteers. This new WPI seems therefore suitable for treatment of OS disturbances and overweight. More investigation is needed in order to confirm the present results on a larger sample size and identify the perfect supplementation dosage and duration. In addition, confounder factors, like physical activity levels, should be checked.

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Appendix A

Variations in the detected parameters among subgroups at the end of the study (statistically significant variations are reported in bold, $p<0.05$).

Category	Parameters	Group A variation (%)	Group B variation (%)	Female variation (%)	Male variation (%)
Antioxidant status	GR (U/l)	3.24	5.65	4.23	0.00
	GPX (U/l)	−21.44	**−15.54**	**−22.19**	−12.32
	Acido urico (mg/dl)	**7.65**	2.34	3.31	0.00
	TAS (mmol/l)	1.56	−2.63	−3.66	0.00
Oxidative damage	TBARS (μmol/g)	22.11	11.98	17.67	0.00
	TBARS (μmol/g creat)	13.11	6.74	−3.72	0.00
	AOPP (μM/Chlor-T-eq)	5.64	0.76	7.10	0.00
	8OHdG (mg/g creat)	−91.19	16.26	−30.09	0.00
Inflammatory state	IL-6 (pg/ml)	1.99	12.12	4.66	0.00
	PCR (mg/dl)	−2.17	−3.32	−16.85	0.00
Metabolic parameters	Glucose (mg/dl)	0.47	5.66	−0.64	0.00
	Insulin (mcUI/ml)	2.69	2.90	9.03	0.00
	HbA1c (%)	−0.12	4.27	0.72	0.00
	HOMA-IR	2.35	5.94	3.91	0.00
Anthropometric parameters	Weight (kg)	3,40	3,47	3,28	**0.00**
	BMI	3.28	**3.42**	**3.14**	**0.00**
	Waist circumference (cm)	3.01	**2.60**	2.16	**0.00**
	Systolic blood pressure (mmHg)	1.86	0.50	2.93	0.00
	Diastolic blood pressure (mmHg)	−3.69	1.44	1.32	0.00
Body compartments	FM (kg)	**8.50**	**9.05**	**6.95**	**0.00**
	FM (%)	**5.05**	**5.68**	**3.40**	**0.00**
	BCM (kg)	1.12	2.36	1.22	0.00
	BCM (%)	1.36	1.91	0.98	**0.00**
Hand grip strength (kg)	ND arm – sitting	2.84	−3.56	−3.51	0.00
	ND arm – standing	−2.04	−2.77	−3.31	0.00
	D arm – sitting	−0.47	−1.90	−3.39	0.00
	D arm – standing	−2.27	−4.30	−5.77	0.00
AF		-7.78	0.26	**−8.16**	**00.00.00**

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